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Outbreak of Infections Caused by *Enterobacter cloacae* Producing the Integron-Associated β-Lactamase IBC-1 in a Neonatal Intensive Care Unit of a Greek Hospital

Georgia Kartali,[1] Eva Tzelepi,[2] Spyros Pournaras,[3] Constantina Kontopoulou,[1] Fanourios Kontos,[3] Danai Sofianou,[1] Antonios N. Maniatis,[3] and Athanassios Tsakris[4]*

Department of Microbiology, Hippokration General Hospital,[1] and Department of Microbiology, Medical School, Aristotle University of Thessaloniki,[4] Thessaloniki, Department of Bacteriology, Hellenic Pasteur Institute, Athens,[2] and Department of Medical Microbiology, University of Thessalia, Larissa,[3] Greece

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Nineteen of 27 ceftazidime-resistant *Enterobacter cloacae* isolates from a neonatal intensive care unit in Thessaloniki, Greece, had genes coding for the novel extended-spectrum β-lactamase IBC-1; 18 of those 19 harbored similar conjugative plasmids and belonged to two distinct genetic lineages. A synergy test with ceftazidime and imipenem enabled us to identify five unrelated bla_{IBC-1}-carrying *E. cloacae* isolates from other wards of the hospital. It seems that this integron-associated gene is capable of dispersing both by clonal spread and by gene dissemination.

Strains of *Enterobacter cloacae* resistant to expanded-spectrum cephalosporins have been implicated in outbreaks of serious infections in neonatal intensive care units (NICUs) (2, 4, 11, 14). In this species stable derepression of the chromosomal Ampler class C β-lactamase is the major cause of resistance to the above drugs (12, 13, 18). However, production of class A extended-spectrum β-lactamases (ESBLs) has also been detected in enterobacters, but their prevalence is generally very low (1, 5, 13). Recently, a novel integron-associated class A β-lactamase with extended-spectrum properties, designated IBC-1, has been described for an *E. cloacae* clinical strain in Greece (3).

Preliminary susceptibility data in our hospital indicated that several infections among preterm neonates in our NICU were due to ceftazidime-resistant *E. cloacae* that usually exhibited cross-resistance to other unrelated antimicrobials. The similar antimicrobial susceptibility patterns of these isolates prompted an investigation to determine whether a limited spread of a single strain existed and also to study the mechanism(s) of resistance to newer β-lactam antibiotics.

During the study period (August 1998 to June 2000), 27 nonrepetitive *E. cloacae* isolates were consecutively collected from clinical specimens of separate premature neonates in the NICU of Hippokration General Hospital. Ceftazidime-resistant nonrepetitive *E. cloacae* isolates that were recovered from separate patients in various other departments of the hospital were also included in the study. Species identification was done by using the Vitek automated identification system (bioMerieux, Marcy l'Etoile, France) and was confirmed with the ATB-GN system (bioMerieux).

Susceptibilities of the isolates to antibiotics were determined by an agar dilution technique (9). The antibiotics used are shown in Table 1. The isolates were screened for the presence of IBC-like ESBLs by placing a disk of ceftazidime (30 μg) at distances of 15 mm (edge to edge) from disks containing imipenem (30 μg), amoxicillin plus clavulanate (20 and 10 μg, respectively), and piperacillin plus tazobactam (75 and 10 μg, respectively). Any enhancement of the zone of inhibition between the ceftazidime disk and that containing any β-lactamase inhibitor was interpreted as presumptive evidence for the presence of an ESBL (1, 7).

Pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA was performed (6), and banding patterns of the strains were compared visually by following the criteria of Tenover et al. (15). In order to investigate the presence of bla_{IBC-1}, a 400-bp internal fragment of the gene was amplified with the primers 5′-TGCATCGGAAAAATTAACCT-3′ (forward) and 5′-AATTTTACGAAAATACTGCG-3′ (reverse), corresponding to the segment 51–450 of the published sequence (3). The conditions were an initial denaturation at 94°C for 5 min; 30 cycles of amplification at 94°C for 25 s, 54°C for 40 s, and 72°C for 50 s; and a final extension at 72°C for 6 min. *E. cloacae* HT9 (3) was used as a positive control in all PCR experiments.

Conjugal experiments were performed as described previously (17). bla_{IBC-1}-bearing strains and transconjugants were analyzed for plasmids by an alkaline lysis procedure (10). For restriction endonuclease analysis, extracted plasmids were digested with 10 U of *Eco*RI. Isoelectric focusing (IEF) of β-lactamases was performed in polyacrylamide gels containing ampholytes (Pharmacia-LKB) covering a pH range from 3.5 to 9.5 (8).

The susceptibilities of the enterobacters that were recovered in the NICU during the study period are presented in Table 1. All 27 *E. cloacae* isolates were resistant to ceftazidime (MIC > 32 μg/ml) while for most of them (16 out of 27) the MICs of cefotaxime, ceftriaxone, and aztreonam were ≤16 μg/ml, indicative of nonoverproduced chromosomal cephalosporinase. By PFGE analysis two major clonal types were detected, in-

* Corresponding author. Mailing address: Department of Microbiology, Medical School, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece. Phone: 30 310 99 90 91. Fax: 30 310 99 91 49. E-mail: atsakris@med.auth.gr.

TABLE 1. Antibiotic susceptibilities of the 27 *E. cloacae* isolates recovered in the NICU

Antibiotic	MIC (μg/ml)				No. of resistant isolates[c]
	Definition of resistance	Range	MIC_{50}[d]	MIC_{90}[e]	
Amoxicillin-clavulanate[a]	>8	>32	>32	>32	27
Piperacillin	>64	128–>128	128	>128	27
Piperacillin-tazobactam[b]	>64	32–>128	128	>128	20
Cefoxitin	>16	>32	>32	>32	27
Ceftazidime	>16	>32	>32	>32	27
Cefotaxime	>32	4–>64	16	>32	9
Ceftriaxone	>32	4–>64	16	>32	10
Aztreonam	>16	8–>32	16	>32	11
Imipenem	>8	≤0.5–2	0.5	1	0
Gentamicin	>4	≤0.5–32	1	4	2
Tobramycin	>4	≤0.5–32	16	32	23
Netilmicin	>16	≤0.5–>64	32	>64	23
Amikacin	>16	≤0.5–>64	32	64	23
Tetracycline	>8	1–>64	>64	>64	26
Trimethoprim	>8	≤0.5–>64	>64	>64	26
Sulfamethoxazole	>256	>512	>512	>512	27

[a] Amoxicillin component of an amoxicillin-clavulanate 2:1 ratio test.
[b] Piperacillin component tested with a fixed concentration of 4 μg of tazobactam per ml.
[c] Resistance rates were defined according to the NCCLS interpretative criteria (9).
[d] MIC at which 50% of the isolates tested are inhibited.
[e] MIC at which 90% of the isolates tested are inhibited.

cluding 12 and 6 isolates (PFGE types I and II, respectively) (Table 2). The remaining isolates belonged to distinct PFGE types that contained one or two isolates. Ceftazidime resistance was transferred at frequencies varying from 3.7×10^{-3} to 6.8×10^{-4} per donor cell by all 18 isolates that belonged to PFGE types I and II; the remaining enterobacters did not transfer ceftazidime resistance. MIC tests confirmed the transfer of resistance to ceftazidime, piperacillin, amikacin, netilmicin, tobramycin, tetracycline, trimethoprim, and sulfamethoxazole in all cases. Plasmids of approximately 85 kb were visualized in all transconjugants that exhibited a high degree of similarity on the basis of their restriction enzyme digestion profiles (Fig. 1).

IEF testing showed production of a β-lactamase with an apparent isoelectric point of 6.9 that corresponded to IBC-1 β-lactamase in all transconjugants. This β-lactamase was also visualized in their donors as well as in a ceftazidime-resistant nontransferable *E. cloacae* isolate that belonged to a separate

TABLE 2. Characteristics of bla_{IBC-1}-carrying isolates

Isolate (PFGE type)	Ward	Date of isolation (mo/day/yr)	Site of isolation	Resistance pattern of clinical isolates[a,b]	Transferable resistance
(I)	NICU	9/7/98	Conjunctiva	PTZ, CAZ, CTX, CRO, ATM, TOB, NET, AMK, TMP	+
2 (II)	NICU	10/18/98	Bronchial tube	CAZ, CTX, CRO, ATM, TOB, NET, AMK, TMP	+
3 (IV)	Surgery 5	1/21/99	Intravascular catheter	PTZ, CAZ, CTX, CRO, ATM, TOB, NET, AMK, TMP	−
4 (II)	NICU	2/19/99	Conjunctiva	CAZ, TOB, NET, AMK, TMP	+
5 (II)	NICU	2/20/99	Pus	CAZ, TOB, NET, AMK, TMP	+
6 (II)	NICU	3/8/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
7 (II)	NICU	3/19/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
8 (I)	NICU	5/17/99	Blood	CAZ, CTX, CRO, ATM, TOB, NET, AMK, TMP	+
9 (I)	NICU	6/18/99	Bronchial tube	PTZ, CAZ, TOB, NET, AMK, TMP	+
10 (I)	NICU	6/20/99	Bronchial tube	CAZ, TOB, NET, AMK, TMP	+
11 (I)	NICU	6/27/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
12 (I)	NICU	6/28/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
13 (II)	NICU	6/30/99	Conjunctiva	PTZ, CAZ, TOB, NET, AMK, TMP	+
14 (I)	NICU	7/17/99	Conjunctiva	CAZ, TOB, NET, AMK, TMP	+
15 (III)	NICU	7/25/99	Blood	PTZ, CAZ, TMP	−
16 (I)	NICU	7/26/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
17 (I)	NICU	7/29/99	Blood	PTZ, CAZ, TOB, NET, AMK, TMP	+
18 (I)	NICU	8/2/99	Blood	PTZ, CAZ, ATM, TOB, NET, AMK, TMP	+
19 (I)	NICU	8/18/99	Blood	PTZ, CAZ, CTX, CRO, ATM, TOB, NET, AMK, TMP	+
20 (V)	Medicine 2	11/15/99	Blood	PTZ, CAZ, CTX, TOB, NET, AMK, TMP	+
21 (VI)	Pediatric ICU	1/14/00	Blood	CAZ, TOB, NET, AMK, TMP	+
22 (VII)	Pediatric ICU	1/27/00	Blood	CAZ, TOB, NET, AMK, TMP	+
23 (I)	NICU	3/20/00	Bronchial tube	PTZ, CAZ, ATM, TOB, NET, AMK, TMP	+
24 (VIII)	Surgery 2	4/25/00	Wound	CAZ, ATM	−

[a] All isolates were resistant to amoxicillin-clavulanate, piperacillin, cefoxitin, sulfamethoxazole, and tetracycline.
[b] Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; NET, netilmicin; PTZ, piperacillin-tazobactam; TMP, trimethoprim; TOB, tobramycin.

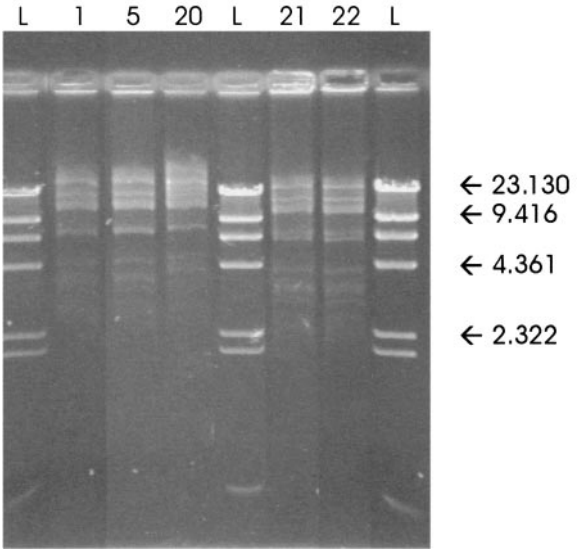

FIG. 1. *Eco*RI restriction digest profiles of five 85-kb plasmids carried by ceftazidime-resistant transconjugants derived from different *E. cloacae* isolates. The origins of the corresponding clinical isolates are shown in Table 2. Lanes L, *Hin*dIII digest of lambda phage.

clonal type (PFGE type III). The gene bla_{IBC-1} was amplified in PFGE type I and II enterobacters and their respective transconjugants as well as in the PFGE type III isolate. No bla_{IBC-1}-specific fragment was detected in the remaining enterobacters.

Synergy tests were used in order to evaluate their efficacy in the detection of bla_{IBC-1}-containing isolates. The test with ceftazidime and imipenem was repeatedly positive for 16 of the bla_{IBC-1}-positive isolates, while in the remaining three cases the test gave enhanced inhibition zones that were not always easy to read; it was negative for the remaining ceftazidime-resistant enterobacters. Synergy tests with ceftazidime and amoxicillin-clavulanate or piperacillin-tazobactam were negative for all *E. cloacae* isolates (Fig. 2).

The synergy test with ceftazidime and imipenem was also used in order to detect *E. cloacae* isolates that express the IBC-1 β-lactamase possibly present in other departments of the hospital. During the study period 34 ceftazidime-resistant enterobacters were recovered from separate patients in various other departments. Five *E. cloacae* isolates derived from four different wards were positive in the synergy test, and PCR and IEF testing confirmed the presence of IBC-1. Their resistance patterns are presented in Table 2. All had macrorestriction patterns distinct from those of one another and from those of isolates recovered in the NICU (PFGE types IV to VIII) (Table 2). Ceftazidime resistance was transferable from three isolates. Their transconjugants exhibited the same resistance profile, and each had one plasmid of the same size (approximately 85 kb) as, and exhibited restriction enzyme profiles similar to, those derived from enterobacters in the NICU.

Genetic analysis of the *E. cloacae* isolates showed that two clones persisted in the NICU during the study period, harboring similar conjugative plasmids that coded for the novel integron-associated ESBL IBC-1. It is of interest that, although the two major *E. cloacae* clones persisted in the NICU for at least 20 months, these strains were not detected outside this unit, at least among IBC-1-expressing *E. cloacae* isolates. Potential reservoirs such as environmental sources were not identified in this study (data not shown). The fact that two *E. cloacae* clones containing the bla_{IBC-1} gene cassettes have been spread in the hospital setting of the NICU might imply that the neonate's gastrointestinal tract became colonized and the transmission occurred via the hands of the medical or nursing staff. However, it is possible that other factors such as overcrowding in the unit and contamination of multidose vials with *E. cloacae* may have contributed to the dissemination of the outbreak strains. After the first episodes of ceftazidime-resistant *E. cloacae* infections guidelines for control of fomites and cross-

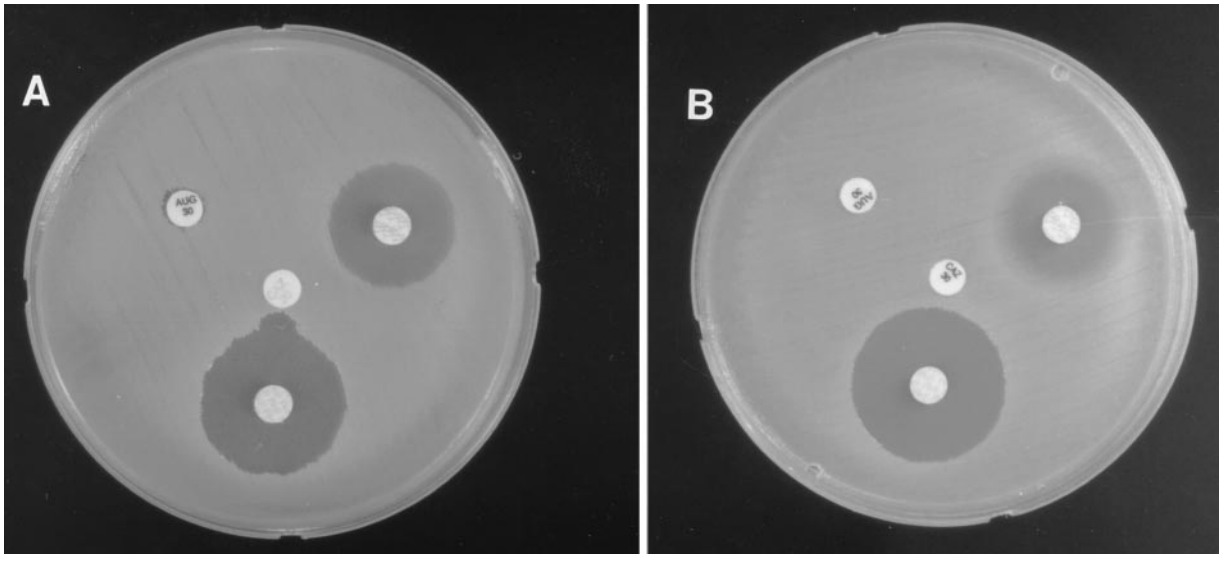

FIG. 2. Synergy tests for representative *E. cloacae* isolates producing IBC-1 enzyme (A) and not producing the enzyme (B). Drug abbreviations on disks: AUG (top left), amoxicillin-clavulanate; IPM (bottom), imipenem; CAZ (center), ceftazidime; TZP (top right), piperacillin-tazobactam. The numbers on the disks are the amounts of drug (in micrograms).

contamination were devised. Thus, all equipment used to care for the patients was decontaminated, and the staff engaged in therapy used disposable gloves and gowns. Indeed, in the NICU there was only one episode of IBC-1-bearing *E. cloacae* from August 1999 through June 2000.

IBC-1 is inhibited more by imipenem and less by β-lactamase inhibitors such as clavulanate and tazobactam (3). The synergy test with ceftazidime and imipenem enabled us to identify ceftazidime-resistant isolates that carried the bla_{IBC-1} gene from other departments of the hospital. In this study, the disks in the synergy test were placed at distances of 15 mm (edge to edge) in order to more precisely recognize ESBLs in derepressed variants of enterobacters (1, 16). It has yet to be determined whether the application of the synergy test could also contribute to the identification of clinical isolates of other gram-negative bacteria producing IBC-like β-lactamases.

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